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Influence of soil temperature and moisture on biochemical biomarkers in earthworm and microbial activity after exposure to propiconazole and chlorantraniliprole

Davorka K. Hackenberger, Goran Palijan, Željka Lončarić, Olga Jovanović Glavaš, Branimir K. Hackenberger*

Department of Biology, University of Osijek, Cara Hadrijana 8A, HR-31000 Osijek, Croatia

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ABSTRACT

Predicted climate change could impact the effects that various chemicals have on organisms. Increased temperature or change in precipitation regime could either enhance or lower toxicity of pesticides. The aim of this study is to assess how change in temperature and soil moisture affect biochemical biomarkers in *Eisenia fetida* earthworm and microbial activity in their excrements after exposure to a fungicide - propiconazole (PCZ) and an insecticide - chlorantraniliprole (CAP). For seven days, earthworms were exposed to the pesticides under four environmental conditions comprising combinations of two different temperatures (20 °C and 25 °C) and two different soil water holding capacities (30% and 50%). After exposure, in the collected earthworm casts the microbial activity was measured through dehydrogenase activity (DHA) and biofilm forming ability (BFA), and in the postmitochondrial fraction of earthworms the activities of acetylcholinesterase (AChE), catalase (CAT) and glutathione-S-transferase (GST) respectively. The temperature and the soil moisture affected enzyme activities and organism's response to pesticides. It was determined that a three-way interaction (pesticide concentration, temperature and moisture) is statistically significant for the CAT and GST after the CAP exposure, and for the AChE and CAT after the PCZ exposure. Interestingly, the AChE activity was induced by both pesticides at a higher temperature tested. The most important two-way interaction that was determined occurred between the concentration and temperature applied. DHA and BFA, as markers of microbial activity, were unevenly affected by PCZ, CAP and environmental conditions. The results of this experiment demonstrate that experiments with at least two different environmental conditions can give a very good insight into some possible effects that the climate change could have on the toxicity of pesticides. The interaction of environmental factors should play a more important role in the risk assessments for pesticides.

1. Introduction

Over the past few decades, there has been extensive research into the toxicity of pesticides and other chemicals on earthworms. Earthworms were given such attention due to their widely known beneficial roles in a number of soil processes and their sensitivity to contaminants, environmental stress and impact on soil quality. Depending on the objective of research, different endpoints are investigated, such as survival, reproduction, avoidance, biochemical biomarkers, gene expression, etc. Most of these studies are conducted in laboratories under the standard exposure conditions. However, climate change could modify the influence that tested chemicals have on organisms. Negative impacts of various pollutants and contaminants may intensify as a consequence of the forecasted temperature increases (1.1–6.4 °C by the year 2100) under the current global warming perspectives (IPCC, 2007). The change in a precipitation regime is also predicted, particularly an increase during winter and a decrease during summer in central Europe (Alcamo et al., 2007). Climate change could have both indirect and direct effects on pesticides (Kattwinkel et al., 2011). The indirect effects include changes in exposure to pesticides due to the shifts in cultivation towards higher latitudes and extension of cultivation periods (Tubiello et al., 2002; Bloomfield et al., 2006). The potential enhancement of volatility and degradation of pesticides could affect their efficiency against pests and, therefore, pesticide application rates could increase (Noyes et al., 2009). This increase of pesticide application could be both in their quantity, but also in the extent of

* Corresponding author.
E-mail addresses: davorka@biologija.unios.hr (D.K. Hackenberger), gpalijan@biologija.unios.hr (G. Palijan), zloncaric@biologija.unios.hr (Ž. Lončarić), ojovanovic@biologija.unios.hr (O. Jovanović Glavaš), hackenberger@biologija.unios.hr (B.K. Hackenberger).

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application area (Koleva and Schneider, 2009). In terms of direct effects, climate change might have an impact on pesticide decomposition and toxicity (Kattwinkel et al., 2011), particularly in the expected alternations in temperature and precipitation (Noyes et al., 2009). It has been shown that the pesticide degradation is dependent on soil moisture and temperature (Kookana et al., 2010). Besides being dependent on the temperature, the toxic effects of pesticides are also linked with other stressors.

Frequently, that results in increasing toxicity with increasing temperature (Kattwinkel et al., 2011). Changes in soil moisture are also associated with differently toxic and environmentally mobile metabolites (Van den Berg et al., 1999). Changes in temperature alter toxicokinetics of toxicants (Lydy et al., 1999), boost an organism's metabolic activity and thus uptake the rates of toxicants (Martikainen and Krogh, 1999; Lima et al., 2015). Soil temperature and soil moisture are key factors influencing growth, survival, fecundity and activity of earthworms (Edwards and Bohlen, 1996) and, indirectly, influencing the earthworm habitat and availability of food (Curry, 2004). Moreover, soil temperature and moisture affect most of the life cycle traits, such as weight, cocoon incubation time, onset of sexual maturity, reproduction and life span. Increase in temperature may accelerate the growth and reproduction rate of earthworms (Uvarov et al., 2011). It has also been shown that soil moisture and temperature have influence on biomarkers in *Aporrectodea caliginosa* earthworms (Booth et al., 2000). Several studies dealing with the influence of soil moisture on effects of different chemicals found a synergistic response (Bindesbøl et al., 2005; Friis et al., 2004; Long et al., 2009; Lima et al., 2011). Toxicity can be different depending on the tested pesticide, temperature and type of soil or a measured endpoint (De Silva et al., 2009). Papers focusing on the combination of toxicity of chemicals and change of both soil moisture and temperature are scarce. Gonzàlez-Alcaraz and van Gestel (2016a, 2016b) studied the bioaccumulation and toxicity of metals/metalloids in earthworms and enchytraeids under different scenarios of climate changes while Bandow et al. (2014) studied the interactive effects of environmental factors and pesticides on collembola. They pointed out that an interaction of environmental factors has not been examined within the chemical risk assessment, but might become more relevant if global climate change accelerates (Bandow et al., 2014).

Beside earthworms, pesticides affect the response of soil microbial communities and possible ecological implications of such exposure raised some serious concerns (Imfeld and Vuilleumier, 2012). The effects of pesticides on soil microbial community became even more complicated with the incorporation of microbial interactions with other non-target organisms such as earthworms. It is known that earthworms could stimulate abundance and activity of pesticide degraders (Liu et al., 2011; Sanchez-Hernandez et al., 2014), while pesticides could have direct effects on both earthworms (Stepić et al., 2013; García-Pérez et al., 2016) and microbes (Imfeld and Vuilleumier, 2012; Petric et al., 2016). The effects of pesticides on soil microbes can be either stimulating or inhibitory.

For the purposes of this research, we chose two pesticides that are classified as nontoxic to earthworms, and their possible adverse effects on earthworms are scarcely studied. One is propiconazole (PCZ), a fungicide from a triazole family, widely used as a systemic foliar fungicide (Konwick et al., 2006). They can be widely distributed into soil after treatment (Wang et al., 2008; Gao et al., 2013). Concerning soil microorganisms, propiconazole stimulated cellulase and invertase activities (Ramudu et al., 2011), while certain negative effects were noted in relation to microbial abundance and soil microbial community structure (Yen et al., 2009). It also decreased the substrate induced respiration, radioactively labelled leucine incorporation (Fernández-Calviño et al., 2017) and phosphatase activity (Kalam and Mukherjee, 2002). The second pesticide used in this research is a novel insecticide chlorantraniliprole (CAP), an anthranilic diamide which has a specific mode of action: it activates ryanodine receptors that can release stored calcium and, hence, cause an impaired muscle contraction regulation (Cordova et al., 2007; Larson et al., 2012). Due to the differential receptor selectivity of CAP between ryanodine receptors in various animal groups, CAP is relatively safe for mammals, birds and fish, but very highly toxic for several aquatic invertebrates (Lavtižar et al., 2016). These features make CAP rapidly replacing other groups of insecticides (Rodrigues et al., 2015).

The aim of this paper is to determine whether changes in temperature and soil moisture have an impact on the toxicity of PCZ and CAP to *Eisenia fetida* earthworm at environmentally relevant concentrations. Therefore, we have made a combination of two temperatures (20 °C and 25 °C) and two soil moisture (30% and 50% WHC) treatments according to Gonzalez-Alcaraz et al. (2015). The impact was examined through the measurement of biochemical biomarkers. Additionally, as the earthworm casts are an important source of substrate for microorganisms in soil (e.g. increased active surface, earthworm cast — microbial hotspot), we have tested the impact of the two pesticides on the microbial activity in the casts through the dehydrogenase activity and the biofilm forming ability. Moreover, we have also tested whether the impact, if any, persists 10 days after the exposure to pesticides in several time steps.

2. Materials and methods

2.1. Organisms

Adult earthworms (*Eisenia fetida*) were obtained from a culture maintained in our laboratory. All earthworms were adults, four months old, with well-developed clitellae and weighted between 250 mg and 450 mg. Prior to each exposure, earthworms were removed from the culture and placed on a damp filter paper overnight to void their gut content.

2.2. Chemicals

All reagents used were of analytical grade. 5,5'dithiobis-2 nitrobenzoic acid (DTNB), acetylthiocholine iodide (AcSChI), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), bovine serum albumin (BSA), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(phenyl) tetrazolium chloride (INT) and Crystal Violet.

The following commercial preparations of pesticides were used — Bumper 25 EC, as a propiconazole formulation (250 g/L propiconazole), and Coragen 20 SC, as a chlorantraniliprole formulation (200 g/L chlorantraniliprole).

2.3. Experimental set-up

Preliminary experiments were conducted in order to determine concentration range that is sublethal and environmentally relevant. In all preliminary experiments biomarkers that are measured in subsequent experiments were also measured. Preliminary experiments comprised a 72-h filter paper test (according to OECD, 1984), and an artificial soil test for 7 and 14 days under the standard conditions (20 °C, 50% WHC).

In the final experiments, four concentrations were applied for each pesticide. Firstly, the highest recommended concentration relevant for field application was calculated according to the label of the commercial formulations. To enable a comparison, an application rates were transformed into $mg_{a.i.}\ kg_{dw\ soil}^{-1}$. An average soil depth of 0.1 m and an average soil bulk density of 1.5 g cm^{-3} was taken into account to facilitate calculations. This environmentally relevant concentrations were labelled as a C3 concentrations. Apart from this concentration, 2-fold and 4-fold lower and 2-fold higher concentrations were also applied. The applied concentrations were: 20.825 μg kg^{-1} (C1), 41.65 μg kg^{-1}(C2), 83.3 μg kg^{-1} (C3) and 166.6 μg kg^{-1} (C4) for propiconazole (PCZ); and 5 μg kg^{-1} (C1), 10 μg kg^{-1}(C2), 20 μg kg^{-1}

(C3) and 40 μg kg^{-1} (C4) for chlorantraniliprole (CAP), respectively. The pesticides were suspended in distilled water and applied to prepared test vessels.

Artificial soil was prepared according to the standard protocols (OECD, 1984) with 70% fine quartz sand, 20% kaolinite clay and 10% sphagnum peat, whereas pH was adjusted to 6.0 ± 0.5 with $CaCO_3$. After mixing, water holding capacity (WHC) was measured and two different moisture levels were calculated (30% and 50% WHC). 50% WHC presents a standard moisture level for an artificial soil test, while 30% WHC presents a low moisture condition. Four hundred grams (dry weight) of artificial soil were placed in each 1-L glass vessel. After the application of pesticides, the soil was left to equilibrate for 6 h. After equilibration period, 8 adult earthworms were placed in the test vessels and covered with perforated lid needed for ventilation. Each treatment was performed in a triplicate; in parallel, controls (K) were run where only distilled water was used. The test vessels were placed at 20 °C and 25 °C in incubators and in the dark.

There were four different temperature and moisture treatments applied: at 20 °C/50% WHC, 20 °C/30% WHC, 25 °C/50% WHC and 25 °C/30% WHC correspondingly. After 7 days of exposure, earthworms were removed from the vessels, washed and placed on a moist filter paper to empty their intestines. The casts were collected for further measurement of dehydogenase activity (DHA) and biofilm forming ability (BFA).

For a post exposure experiment, earthworms were exposed to the same concentrations and duration of exposure. After 7 days, the earthworms were removed from the soil treated with pesticides and transferred to the clean artificial soil. Earthworms were sampled on the days 1, 3, 5, and 10 after the exposure to pesticides was terminated. Earthworms were then removed from the vessels, washed with distilled water and placed on a moist filter paper to void their intestines. Casts were collected for further measurement of dehydogenase activity (DHA) and biofilm forming ability (BFA).

2.4. Sample preparation

Earthworms were individually weighted and homogenized in a cold sodium phosphate buffer (0.1 M, pH 7.4) (1:5/w:v) with a Potter—Elvehjem homogenizer. The homogenates were then centrifuged at 9000 x *g* for 30 min and 4 °C to yield the postmitochondrial fraction. Aliquots of the supernatant were stored at − 80 °C until further analysis.

The AChE activity was determined according to the method of Ellman et al. (1961), spectrophotometrically at 412 nm. The reaction medium (1500 μL) consisted of a sodium phosphate buffer (0.1 M, pH 7.2), DTNB (1.6 mM), AcSChI (156 mM) and sample (S9). The specific enzymatic activity was expressed as nmol of acetylthiocholine hydrolysed per min per mg of protein calculated with a molar extinction coefficient of 13.6 mM^{-1} cm^{-1}.

The CAT activity was measured spectrophotometrically at 240 nm following the method developed by Claiborne (1985). The reaction medium consisted of a sodium phosphate buffer (0.1 M, pH 7.2), hydrogen peroxide (0.019 M) and sample (S9). The specific enzymatic activity was expressed as nmol of degraded hydrogen peroxide per min per mg of protein calculated with a molar extinction coefficient of 42.6 M^{-1} cm^{-1}.

The glutathione S-transferase activity was determined spectrophotometrically at 340 nm according to Habig et al. (1974). The reaction mixture contained CDNB (1 mM), GSH (25 mM), and sample (S9). The specific enzymatic activity was expressed as nmol of S-(2, 4-dinitrophenyl) glutathione (DNPG) generated per min per mg of protein calculated with a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

The total content of proteins per earthworm was measured using the Bradford method (Bradford, 1976). The dehydrogenase activity (DHA) of microbial community was determined by the use of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(phenyl) tetrazolium chloride) incubation test (Garcia et al., 1997). Excrements from eight earthworms per treatment were collected after they emptied their intestines into sterile Petri plates. The excrements were collected in sterile, pre-weighted, 2 ml plastic test tubes with caps. The tubes were weighted and filled with 450 μl of sterile distilled water. After vortexing, 200 μl of each sample was transferred into 48 well plates for a biofilm development test. The rest of the sample was amended with 250 μl of 1 M TRIS buffer pH 7.4 and 125 μl 0.04% INT solution. After brief vortexing, the samples were incubated for 24 h in the dark at 40 °C. Then the samples were centrifuged at 10,000 × *g* for 5 min at room temperature (23 ± 1 °C). The supernatant was replaced with 1 ml of methanol and briefly vortexed. The formazan was extracted for 24 h at the room temperature in the dark, centrifugation was repeated and supernatant absorbance was measured at 480 nm spectrophotometrically.

The biofilm forming ability (BFA) of microbial community was determined by incubation of 200 μl of the suspension prepared from the earthworms excrements. Clear, sterile, polystyrene 48 well plates with lids were incubated for 60 h at 25 °C in a humidified incubator. After incubation the wells were stained with 20 μl of Crystal Violet 0.1% final concentration for 15 min at room temperature (O'Toole, 2011). The excessive stain was washed three times with distilled water and plates were left to air dry. The stain was dissolved in 1 ml of 96% ethanol while absorbance was measured at 588 nm spectrophotometrically using Shimadzu UV-1601 spectrophotometer.

2.5. Data presentation and statistical analysis

All data analyses were performed with the use of the statistical software R version 3.4.0 (R Development Core Team, 2017) and RStudio (RStudio Team, 2016).

Prior to the statistical analysis, the data were tested for normality using the Shapiro–Wilk test and homogeneity of variance with Levene test. As no statistically significant deviation from normality was observed, a three-way ANOVA was used to detect whether pesticide concentration, temperature, soil moisture and their interaction affected enzyme activities. Simple main effects for significant interactions were tested by evaluating contrasts across one-factor levels while keeping other interaction factors fixed. For testing significant interactions, the function *testInteractions()* in R package *phia* was used (De Rosario-Martinez, 2015). The analyses of controls between treatments were done using the two-way ANOVA with Tukey's significant honestly difference test as a post hoc.

One way ANOVA with a post hoc Dunnett's test was used to analyse differences in enzyme activities between concentrations and control within a temperature x moisture treatment.

3. Results

All applied doses were sublethal and no mortality was recorded during the experiments. Also, there were no visible morphological changes in the exposed earthworms.

The interaction with the soil moisture was prevailing for AChE and CAT activities ($p < 0.001$) and the interaction with temperature for GST activity ($p < 0.001$). At lower soil moisture the activities of AChE and CAT were significantly higher at 20 °C. The effect on GST was different, with activities significantly higher at lower tested temperature (Fig. 1).

3.1. Effects of pesticide/environmental factors combination on molecular biomarkers

3.1.1. Catalase (CAT) activity

The CAT relative activities after exposure to PCZ and CAP and different temperature/moisture treatments are given in Fig. 2 while the results of the three-way ANOVA are shown in Table 1.

The three-way ANOVA of CAT activity in various treatments with

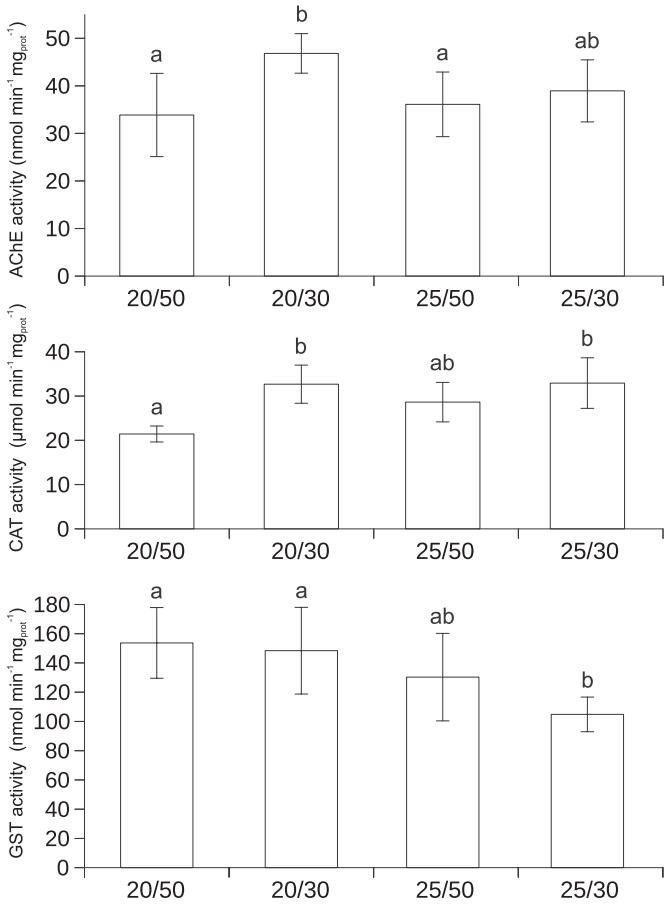

Fig. 1. Specific enzymatic activities of control groups under different temperature and moisture treatments (e.g. 20/50 temperature/soil moisture). The values are given as a mean ± sd. Bars with different letter are statistically significantly (two-way ANOVA, $p < 0.01$).

PCZ pointed out a significant interaction of concentrations, temperature and soil moisture ($p < 0.01$) and a significant interaction between concentration and temperature ($p < 0.01$) (Table 1). A higher increase in the CAT activity was observed at concentrations C2 and C3 at 25 °C in comparison with the CAT activity at 20 °C (Fig. 2). After the exposure to PCZ, lower soil moisture led to a significant decrease in the CAT activity under 20 °C at all concentration levels (up to 14.4%), but only at the first three concentration levels under 25 °C (up to 24.2%) (Fig. 2). The increased CAT activity was measured at all concentration levels under 20 °C/50% WHC treatment and at last two concentration levels under 25 °C/50% and C4 under 25 °C/30% WHC treatment (Fig. 2).

Results of the three-way ANOVA on the CAT activity after the exposure to various CAP concentrations demonstrated that the CAT activity was significantly different with respect to concentration, temperature and soil moisture ($p < 0.001$) (Table 1). Similarly to the exposure to PCZ, there was no significant interaction between the applied concentration levels and soil moisture. A significant interaction occurred between temperature and soil moisture ($p < 0.001$). The catalase activity was different across temperatures at 30% WHC, and different across soil moisture at 25 °C ($p < 0.001$). In 25 °C treatments the catalase activity did not change significantly when compared to the control.

3.1.2. Acetylcholinesterase activity

Results of the three-way ANOVA of the AChE activity, as a result of exposure to CAP and PCZ, showed that all three tested factors, i.e. concentration, temperature and soil moisture, were individually significant ($p < 0.001$). From two-way interactions only concentration and temperature were statistically significant ($p < 0.001$) (Table 1). There was statistically significant three-way interaction among tested variables after the PCZ exposure ($p < 0.01$), but not after CAP exposure. After exposure to both pesticides AChE activity significantly increased with increasing concentrations at both 25 °C treatments and at 20/50 treatment. The only AChE activity decrease, although not significant, was at both 20/30 treatments (Fig. 2).

3.1.3. Glutathione S–transferase

The three-way analysis of variance for the GST activity, as a result of the exposure to CAP and PCZ, indicated that all three tested factors, i.e. concentration, temperature and humidity, individually were significant ($p < 0.05$) (Table 1). There was no significant two-way interactions in treatments with PCZ, while after CAP exposure there was a statistically significant interaction between the applied concentrations and temperature ($p < 0.001$) as well as a significant interaction between the concentrations and soil moisture ($p < 0.05$). The three-way interaction between the tested factors was significant only for CAP ($p < 0.01$).

After the exposure to PCZ, the GST activities increased under all treatments and concentrations when compared to control (Fig. 2). After the exposure to CAP, the GST activities under 20 °C treatments showed either a non-significant decrease or the values similar to the corresponding control. The highest decrease of the GST activity was 17.3% (50% WHC) and 16.8% (30% WHC). On the other hand, at a higher temperature the GST activities increased when compared to the control at the first three concentration levels (Fig. 2).

3.1.4. Post-exposure experiment

All the measured enzyme activities in the post-exposure experiment are shown in Fig. 3. The changes observed during ten days post exposure differed between the treatments. When the values obtained immediately after 7 days of exposure were compared with the ten days post-exposure values, some trends were observed (Fig. 3). Namely, an increase in enzyme activities occurred mostly under 25 °C/50% WHC and 20 °C/30% WHC treatments. The increase was measured for AChE, CAT and GST activities after CAP exposure and CAT and GST activities after the PCZ exposure. On the other hand, a decrease occurred under 25 °C/30% WHC and 20 °C/50% WHC treatments for AChE, CAT and GST after the CAP exposure. While after the exposure to PCZ a decrease in the AChE activity occurred under all four treatments and under three treatments for GST (20 °C/50% WHC, 20 °C/30% WHC and 25 °C/30% WHC), the CAT activities have not changed much between these two time points.

3.2. DHA and BFA activity

Chlorantraniliprole also stimulated the dehydrogenase activity at 20 °C, but at 25 °C stimulation occurred only at 25 °C/30% WHC and C1 and C4 pesticide concentrations (Fig. 4). The biofilm forming ability was always higher compared to the control at lower soil moisture conditions (Fig. 4). It is important to notice that CAP at higher soil moisture level decreased BFA for up to 30% compared to the control. Regarding the biofilm formation per unit of activity of the gut microbial community BFA at lower temperature was inversely related to CAP concentration, while it was proportional to the CAP concentration at higher temperature (Fig. 4). At all tested CAP concentrations, the effect of CAP on BFA per unit of activity of microbial community from earthworm excrements was highest at the least favourable environmental conditions tested (higher temperature, lower soil moisture) and at the higher CAP concentration tested (C3, C4). The coefficients of determination between the microbial activity and biofilm formation were 0.96, 0.01, 0.88 and 0.08 for 20 °C/50% WHC, 20 °C/30% WHC, 25 °C/30% WHC and 25 °C/50% WHC treatments, respectively.

The propiconazole stimulated dehydrogenase activity (DHA) at all tested treatments and pesticide concentrations, except at lower temperature treatments at the highest concentration (Fig. 4). The highest

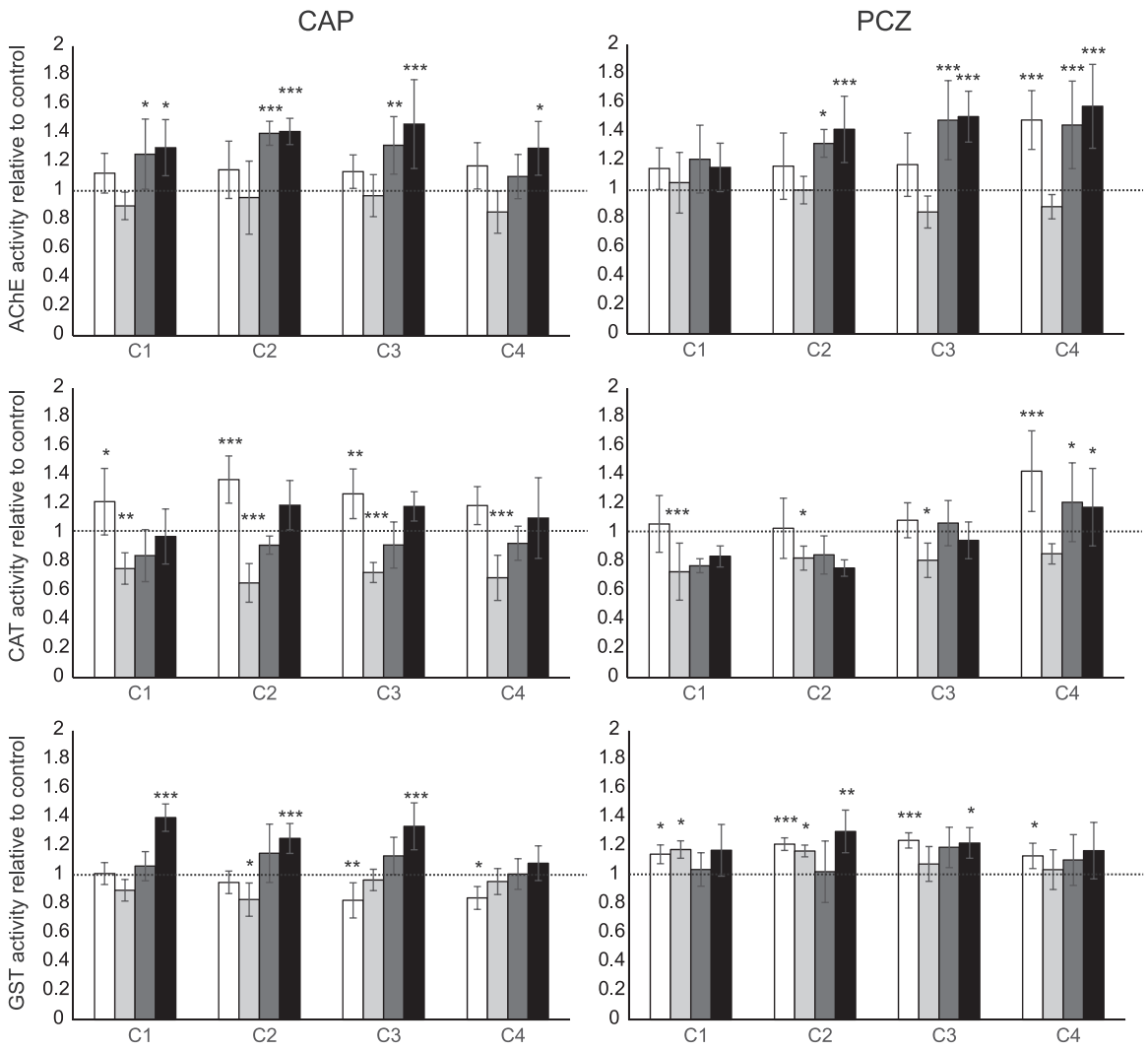

Fig. 2. Activities of AChE, GST and CAT expressed relative to the respective control after the exposure to pesticides. A dotted line presents the control value. Asterisks present statistically significant differences to control (one-way ANOVA): * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). 20 °C/50 WHC (□), 20 °C/30 WHC (▒), 25 °C/50 WHC (▓) and 25 °C/30 WHC (■).

stimulation occurred at the lowest PCZ concentration. The biofilm forming ability (BFA) was similarly stimulated by the PCZ except at lower temperature treatments at the highest concentration, but also at C3 and higher temperature treatments (Fig. 4). Regarding the biofilm formation per unit of activity of the gut microbial community, PCZ at the increased soil temperature decreased the relative biofilm forming ability (Fig. 4). At a lower soil temperature this occurred only at the highest PCZ concentration. The coefficients of determination (r^2) between the microbial activity (DHA) and biofilm formation were 0.79, 0.98, 0.61 and 0.36 for 20 °C/50% WHC, 20 °C/30% WHC, 25 °C/30% WHC and 25 °C/50% WHC treatments, respectively.

Table 1
Summary of the effects of pesticides and temperature/moisture treatments on various *Eisenia fetida* biomarkers. F values for the three-way ANOVA on the effect of pesticide concentration (C), temperature (T) and moisture (M) on the AChE, CAT and GST activity after 7 days exposure, df- degrees of freedom. Any statistically significant differences of various levels are marked with an asterisk.

	CAP				**PCZ**			
	df	AchE	CAT	GST	df	AchE	CAT	GST
Concentration (C)	4	9.18***	2.068	2.9*	4	13.68***	21.53***	9.08***
Temperature (T)	1	37.65***	51.56***	6.3*	1	38.33***	29.04***	156.55***
Moisture (M)	1	28.340***	37.36***	9.86**	1	14.33***	24.63***	30.07***
C × T	4	6.24***	2.54*	8.57***	4	9.19***	3.79**	0.99
C × M	4	0.77	1.07	2.82*	4	1.87	1.69	1.79
T × M	1	0.02	49.15***	2.47	1	0.05	0.002	0.49
C × T × M	4	1.942	10.35***	4.00**	4	4.71**	4.18**	0.76

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

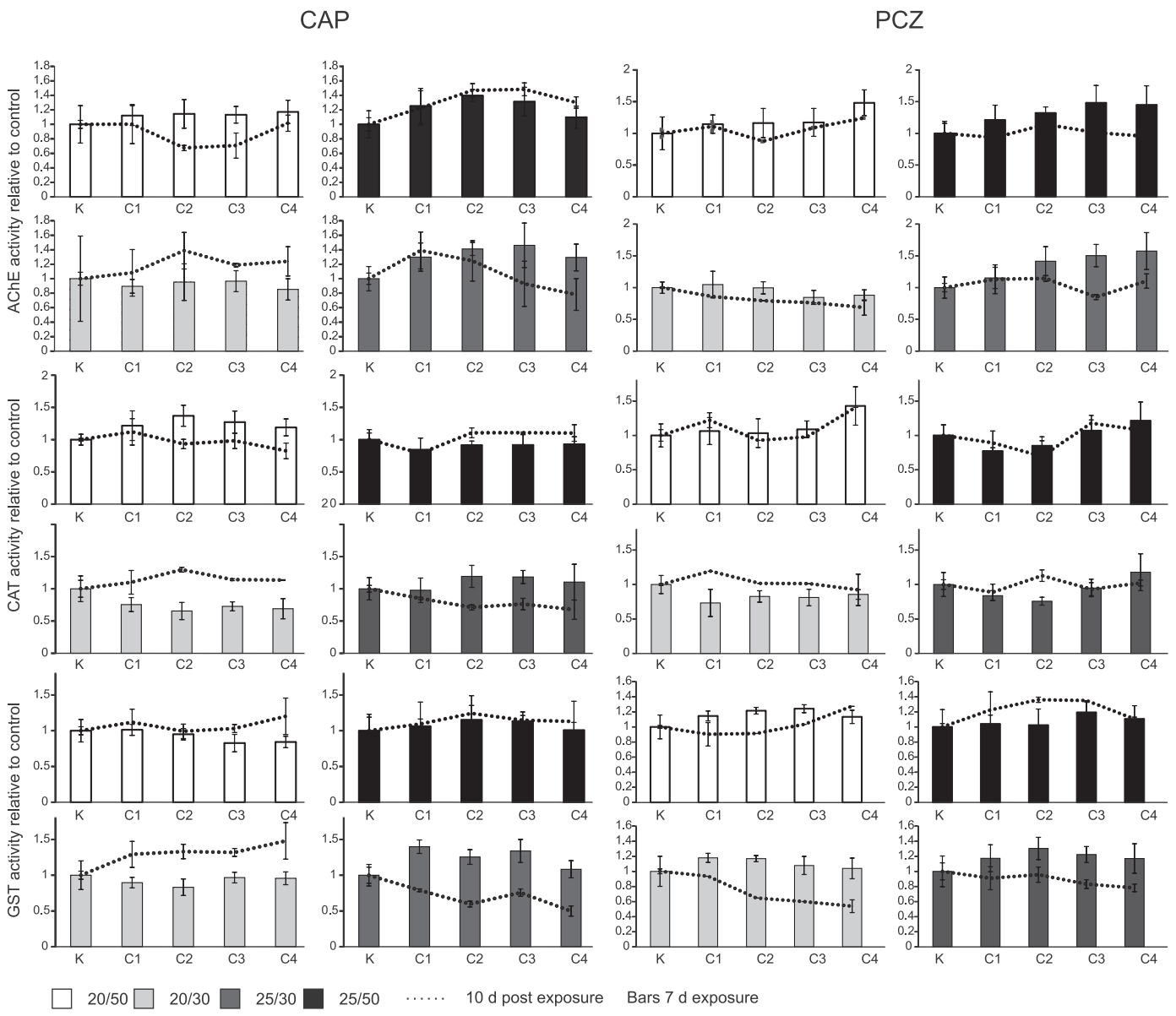

Fig. 3. Biomarker activities expressed relative to the control after 7 days of exposure and after 10 days in clean soil. The values are given as a mean ± sd. Bars - 7 days of exposure, dotted line (—) - 10 days in clean soil.

4. Discussion

4.1. Effect of temperature and soil moisture

Booth et al. (2000) found a significant three-way interaction effect of soil, moisture content and temperature on the earthworm growth, which means that the effect of one parameter cannot be interpreted separately. In this experiment a statistically significant interaction between concentration, temperature and soil moisture was observed after the exposure to both pesticides (Table 1). Papers on the interaction of two environmental variables and a stressor such as a pesticide are very scarce. Nonetheless, in studies that are published it is pointed out that due to a climate change and the usage of pesticides in different climatic regions toxicity should be tested with different sets of environmental variables for a more realistic risk assessment. In the review published by Laskowski et al. (2010), it was stated that in half of the cases natural conditions significantly changed the effects toxicants had on tested organisms. When the influence of one environmental variable and pesticide were investigated, the endpoints were mainly survival and reproduction. The obtained results varied depending on a number of parameters (De Silva et al., 2009). Some pesticides expressed higher toxicity at higher temperatures (Garcia, 2004), while for others toxicity was lower at a higher temperature (Garcia, 2004; De Silva et al., 2009; Lima et al., 2015; Bednarska et al., 2017). Similar results were found for metals, where higher temperature led to a synergistic effect (Khan et al., 2007). This synergism was explained with an enhanced metabolism (Khan et al., 2007), which can lead to a stronger uptake of pesticides through the skin (Römbke et al., 2007). A lower toxicity at a higher temperature could be the result of temperature effects on the chemical stability that leads to a reduced exposure concentration over time (De Silva et al., 2009). We found a statistically significant interaction between concentration and temperature for almost all measured biomarkers (Table 1). However, when only controls are compared, soil moisture was singled out as statistically significant for AChE and CAT, and for GST it was temperature (Fig. 1). Increasing temperature and decreasing moisture induced the CAT activity, which has been observed in different organisms (Khessiba et al., 2005; Tu et al., 2012). However, contrary to findings of Booth et al. (2000), this GST activity increased with increasing temperature and decreased with increasing moisture level, and we observed a decrease of activity in the control group with

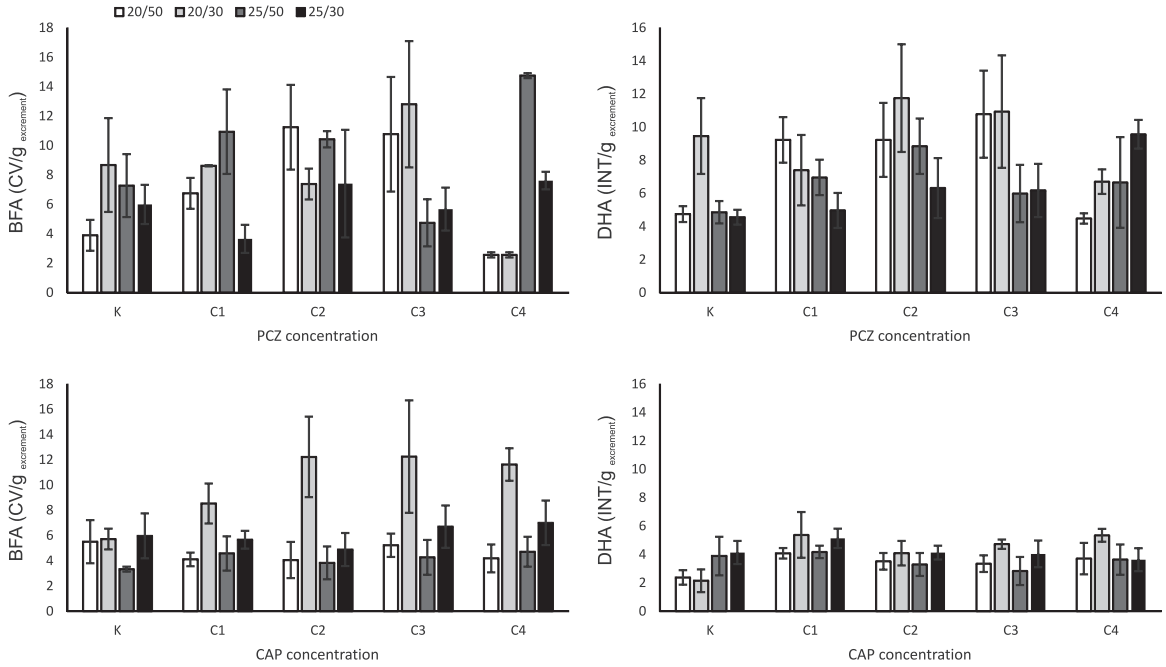

Fig. 4. Results of dehydrogenase activity (DHA) and biofilm formation ability (BFA) expressed as an absorption of INT and crystal violet per g of excrement after 7 days of exposure to chlorantraniliprole (CAP) and propiconazole (PCZ) under different temperature and soil moisture conditions. The values are given as a mean ± SE.

higher temperature and moisture levels (Fig. 1). The reason for this might be the fact that their experiment was conducted only up to 20 °C. Booth et al. (2000) also found that the ChE activity increased with temperature, but that soil moisture had no effect. In our research the AChE activity slightly increased with temperature and was higher under lower moisture levels at the same temperature treatment because a mild significant interaction occurred between temperature and soil moisture (two-way ANOVA, $p < 0.05$) (Fig. 1).

4.2. Effects of chlorantraniliprole

Chlorantraniliprole (CAP), as a novel insecticide with low toxicity to non-target organisms, has a tendency to become widespread and used in high quantities. However, the research on its toxicity is scarce. Only recently Lavtižar et al. (2016) conducted a study on reproduction and survival of *Enchytraeus crypticus* (Oligochaeta) after the exposure to CAP and found that it was not affected up to 1000 $mg_{CAP}/kg_{dw\ soil}$, a much higher concentration that we used in this study. On the other hand, Shaikh et al. (2016) found the effect of CAP on digestive enzymes of *Eudrilus eugeniae* earthworm, which represents a stress in organism and disturbance in the biochemical metabolism. The influence of CAP in our experiment was more pronounced at higher temperature and both moisture levels for AChE and GST (Figs. 1, 2). The three-way ANOVA revealed a significant interaction between concentration and temperature (Table 1). Although the inhibition of the AChE activity is a commonly used biomarker of exposure, in our experiment the AChE activity increased after the exposure to CAP. It is in line with the other studies on different organisms where the exposure to CAP also induced the AChE activity (Rodrigues et al., 2015; Jia et al., 2016). The only inhibition of the AChE activity occurred at 20 °C/30% WHC after the exposure to both CAP and PCZ. As CAP is a calcium homeostasis disruptor in the central nervous system, it is not expected to inhibit AChE as an anticholinergic compound (Li et al., 2011). Although it occurs quite often, the increase of the AChE activity is rarely explained, however, it seems to be connected to the oxidative stress and alternations in the intracellular ion homeostasis (Qi et al., 2013; Mrdaković et al., 2016). An increased AChE activity was also described as a marker of exposure to apoptosis-inducing substances, i.e. a response of cells undergoing apoptosis (Zhang et al., 2002; Jiang et al., 2012), and could be due to a phenomenon of overcompensation, or could be correlated to tissue inflammation (Gambardella et al., 2014). On the other hand, the CAT activity was both induced and decreased depending on the temperature/moisture combination (Fig. 2), for CAT there was a significant two way interaction between temperature and soil moisture and a three way interaction between concentration, temperature and soil moisture (Table 1). Rodrigues et al. (2015) also found a decrease of CAT and GST activity after a short-term exposure to low concentrations of CAP. As catalase is an anti-oxidant enzyme that scavenges H_2O_2 to water and oxygen, the inhibition of the CAT activity may suggest that the capacity to scavenge ROS is reduced. Some authors have suggested that the inhibition of CAT could lead to an accumulation of excess H_2O_2 and could cause a greater oxidative damage during a longer exposure (Wang et al., 2016).

CAP had a more uniform effect on the microbial activity in comparison to PCZ in the applied concentrations. At a lower tested temperature, it always stimulated DHA but at a higher temperature the effects were often negative, especially in the 25 °C/50% WHC treatment. On the other side, the biofilm forming ability was almost always inhibited at higher soil moisture levels (except at C4) and it was always stimulated by lower soil moisture levels. The negative effect on the biofilm formation of CAP has not been established in the literature, yet. Nevertheless, it is known that some heterocycles with amide groups, such as in CAP, have an anti-biofilm activity (Reddy et al., 2015). Such direct negative effect on biofilm formation at increased soil moisture levels could have adverse effects on soil organic matter transformation, as biofilms represent dominant bacterial life form in the terms of abundance and activity (Costerton, 2007). Similar negative effects of different pesticides on biofilms were noted in the aquatic ecosystems at environmentally relevant concentrations (Ricart et al., 2009, 2010). Ricart et al. (2009) reported that functioning of the biofilm community was restored after one month of the experiment. In the future, the long term effects of the CAP on the soil biofilm communities should be investigated.

4.3. Effects of propiconazole

The only research directly studying the influence of PCZ to earthworms showed that it did not affect the earthworm immune system (Bunn et al., 1996), whilst other parameters have not been investigated. Our results showed that the effect of PCZ on CAT activity in *E. fetida* was similar to the effect of CAP. Namely, the highest activity was measured under 20 °C/50% WHC treatment with the strongest increase at C4 concentration. Other treatments seem to completely inhibit the CAT activity at lower concentrations of PCZ (Fig. 2). There was a significant interaction between concentration and temperature and a significant interaction between all three factors (Table 1). A reduced CAT activity has been reported after the exposure to PCZ as a result in an increase of cellular H_2O_2 (Zhang et al., 2008; Nwani et al., 2010; Tabassum et al., 2016). An oxidative stress that can be induced by PCZ has been associated with binding of PCZ to cytochromes and as a consequence promoting the generation of ROS (Li et al., 2010a; Tabassum et al., 2016). Additionally, PCZ metabolites could be a potent source of ROS that can cause damage to molecules resulting in a reduced enzymatic activity (Li et al., 2010b; Tabassum et al., 2016). The AChE activity was inhibited only at higher concentrations of PCZ and 20 °C/30% WHC treatment; in all other treatments the AChE activity was induced. The interactions between factors were the same as for CAT. As mentioned for CAP, the increase of the AChE activity could be a marker of the exposure to apoptosis-inducing substances. Indeed, Gao et al. (2013) showed that exposure to PCZ induced cell pyknosis and cytoplasm deep stains, as an indication of possible cell apoptosis. Results of the GST activity indicated an oxidative stress, which organisms could cope better with under lower concentrations. A decrease of the GST activity at higher concentrations of PCZ has been observed in *Salmo trutta* where GST showed a bell shaped induction (Egaas et al., 1999). We were unable to establish any interactions between the factors, as it was the case for the other two enzymes.

Propiconazole stimulated DHA in earthworms excrements at all tested concentrations but not at both tested temperatures. A combination of a lower temperature and the highest tested PCZ concentration reduced the DHA. At the same time, a higher temperature and C4 resulted in an increased DHA, which is in agreement with the other authors who established increased DHA at the same PCZ concentration and similar temperature levels (28 ± 4 °C) (Ramudu et al., 2015). It is not clear why lower temperature decreased DHA. Soil DHA was not considerably different at 20 °C compared to the 25 °C (Wolińska and Stępniewska, 2012), while it is generally accepted that earthworm gut transition increases the enzymatic activity. The biofilm formation was related to the DHA with an increased biofilm formation almost completely coinciding with the increased DHA. This was an expected result as the biofilm formation is density-dependent while DHA is often positively related to microbial biomass (Tarradellas et al., 1996). Nevertheless, PCZ exerted dominantly negative effects when the relative biofilm production standardized over microbial activity was investigated, i.e. although the microbial community was active, it produced less biofilm then the control. Obviously, PCZ has a stimulating effect on the activity of microorganisms but their relative capability of producing biofilms, i.e. of colonizing new soil surfaces, was decreased with the largest inhibition at the highest applied concentration.

Although pore water is the main route of exposure for soil-dwelling organisms (Lavtižar et al., 2016) and soil solution of PCZ was found to be higher at higher moisture levels (Roy et al., 2000) in our study more negative effects were found under lower moisture levels. Perhaps the stress induced by lower moisture overcompensated the stress induced by pesticide when low concentrations were applied. Puurtinen and Martikainen (1997) explained lower benomyl toxicity for *Enchytraeus sp*. at high soil moisture with a decreased bioavailability as a consequence of compound degradation coupled with an increased adsorption to the solid phase. As with temperature, increasing soil moisture has been found to decrease, increase or have no effect on the toxicity of soil invertebrates (Puurtinen and Martikainen, 1997; Lima et al., 2011; Gonzalez-Alcaraz and van Gestel, 2016a, 2016b). In one of the rare studies including soil moisture, temperature and pesticide exposure, toxicity of pyrimethanil to two collembola species was found to be the highest at the lowest water holding capacity and a higher tested temperature (Bandow et al., 2014). In our study a three-way interaction of pesticide concentration, temperature and soil moisture was significant for both pesticides. The combination of lower soil moisture and higher temperature levels, together with higher concentration of the applied pesticide exerted a stronger biomarker response.

5. Conclusion

The results of our experiment did not indicate a unique response to either influence of temperature or moisture to the toxicity of the tested pesticides, although there was a significant three-way interaction between them. Overall, for the measured biomarkers, the interaction between concentration and temperature was more important than interactions between the other factors. A significant interaction of three factors (concentration, temperature and soil moisture) was present for both applied pesticides. Another interesting result is an increased AChE activity obtained after the exposure to both pesticides at higher temperature levels, which should get more attention in the future research. Also, the microbial dehydrogenase activity and biofilm formation were unequally affected by PCZ and CAP with the interaction of temperature and soil moisture. The results of this experiment demonstrate that experiments with at least two different environmental conditions provide a good insight into possible effects that climate change could have on the toxicity of pesticides. The interaction of environmental factors should gain a larger role in the risk assessments of pesticides.

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References

Alcamo, J., Moreno, J.M., Novaky, B., Bindi, M., Corobov, R., Devoy, R.J.N., Giannakopoulos, C., Martin, E., Olesen, J.E., Shvidenko, A., 2007. Europe. Climate change 2007: impacts adaptation and vulnerability. In: Parry, M.L., Canziani, O.F., Palutikof, J.P., van der Linden, P.J., Hanson, C.E. (Eds.), Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom, pp. 541–580.

Bandow, C., Coors, A., Karau, N., Römbke, J., 2014. Interactive effects of lambda-cyhalothrin, soil moisture and temperature on *Folsomia candida* and *Sinella curviseta* (Collembola). Environ. Toxicol. Chem. 33, 654–661.

Bednarska, A.J., Choczyński, M., Laskowski, R., Walczak, M., 2017. Combined effects of chlorpyriphos, copper and temperature on acetylcholinesterase activity and toxicokinetics of the chemicals in the earthworm *Eisenia fetida*. Environ. Poll. 220, 567–576. http://dx.doi.org/10.1016/j.envpol.2016.10.004.

Bindesbøl, A., Holmstrup, M., Damgaard, C., Bayley, M., 2005. Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*. Environ. Toxicol. Chem. 24, 1462–1467.

Bloomfield, J.P., Williams, R.J., Gooddy, D.C., Cape, J.N., Guha, P., 2006. Impacts of climate change on the fate and behaviour of pesticides in surface and groundwater—a UK perspective. Sci. Total Environ. 369 (1–3), 163–177.

Booth, L., Heppelthwaite, V., Mc Glinchy, A., 2000. The effect of environmental parameters on growth cholinesterase activity and glutathione S-transferase activity in the earthworm (*Aporrectodea caliginosa*). Biomarkers 5 (1), 46–55. http://dx.doi.org/10.1080/135475000230532.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein-dye landing. Anal. Biochem. 72, 248–254.

Bunn, K., Thompson, H., Tarrant, K., 1996. Effects of agrochemicals on the immune systems of earthworms. Bull. Environ. Contam. Toxicol. 57, 632–639. http://dx.doi.org/10.1007/s001289900237.

Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods in Oxygen Radical Research. CRC Press, Boca Raton, FL, pp. 283–284.

Cordova, D., Benner, E.A., Sacher, M.D., Rauh, J.J., Sopa, J.S., Lahm, G.P., Selby, T.P.,

Stevenson, T.M., Flexner, L., Gutteridge, S., Rhoades, D.F., Wu, L., Smith, R.M., Tao, Y., 2007. The novel mode of action of anthranilic diamide insecticides: ryanodine receptor activation. Synth. Chem. Agrochem VII, 223–234. http://dx.doi.org/10.1021/bk-2007-0948.ch017.

Costerton, J.W. (Ed.), 2007. The Biofilm Primer. Springer, Berlin.

Curry, J.P. 2004. Factors affecting the abundance of earthworms in soils. In: Earthworm Ecology. CRC Press, pp. 401–424.

De Rosario-Martinez, H. 2015. phia: Post-Hoc Interaction Analysis. R package version 0.2-1. ⟨https://CRAN.R-project.org/package=phia⟩.

De Silva, P.M.C.S., Pathiratne, A., van Gestel, C.A.M., 2009. Influence of temperature and soil type on the toxicity of three pesticides to *Eisenia andrei*. Chemosphere 76, 1410–1415.

Edwards, C.A., Bohlen, P.J., 1996. Biology and Ecology of Earthworms, 3rd ed. Springer, London, pp. 426.

Egaas, E., Sandvik, M., Fjeld, E., Källqvist, T., Goksøyr, A., Svensen, A., 1999. Some effects of the fungicide propiconazole on cytochrome P450 and glutathione S-transferase in brown trout (*Salmo trutta*). Comp. Biochem. Physiol. C 122, 337–344.

Ellman, G.L., Courtney, K.D., Andres, V., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Fernández-Calviño, D., Rousk, J., Bååth, E., Bollmann, U.E., Bester, K., Brandt, K.K., 2017. Ecotoxicological assessment of propiconazole using soil bacterial and fungal growth assays. Appl. Soil Ecol. 115, 27–30. http://dx.doi.org/10.1016/j.apsoil.2017.03.009.

Friis, K., Damgaard, C., Holmstrup, M., 2004. Sublethal soil copper concentrations increase mortality in the earthworm *Aporrectodea caliginosa* during drought. Ecotoxicol. Environ. Saf. 57, 65–73.

Gambardella, C., Mesarič, T., Milivojević, T., Sepčić, K., Gallus, L., Carbone, S., Ferrando, S., Faimali, M., 2014. Effects of selected metal oxide nanoparticles on *Artemia salina* larvae: evaluation of mortality and behavioural and biochemical responses. Environ. Monit. Assess. 186, 4249–4259. http://dx.doi.org/10.1007/s10661-014-3695-8.

González-Alcaraz, M.N., Tsitsiou, E., Wieldraaijer, R., Verweij, R.A., van Gestel, C.A., 2015. Effects of climate change on the toxicity of soils polluted by metal mine wastes to *Enchytraeus crypticus*. Environ. Toxicol. Chem. 34 (2), 346–354.

Gao, M., Song, W., Zhang, J., Guo, J., 2013. Effect on enzymes and histopathology in earthworm (*Eisenia foetida*) induced by triazole fungicides. Environ. Toxicol. Pharmacol. 35 (3), 427–433. http://dx.doi.org/10.1016/j.etap.2013.02.003.

Garcia, C., Hernandez, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plant Anal. 28, 123–134. http://dx.doi.org/10.1080/00103629709369777.

Garcia, M.V.B., 2004. Effects of Pesticides on Soil Fauna: Development of Ecotoxicological Test Methods for Tropical Regions. Ecology and Development Series, vol. 19 19 University of Bonn, Germany.

García-Pérez, J.A., Alarcón, E., Hernández, Y., Hernández, C., 2016. Impact of litter contaminated with glyphosate-based herbicide on the performance of *Pontoscolex corethrurus*, soil phosphatase activities and soil pH. Appl. Soil Ecol. 104, 31–41. http://dx.doi.org/10.1016/j.apsoil.2016.03.007.

González-Alcaraz, N.M., van Gestel, C.A.M., 2016a. Metal/metalloid (As, Cd and Zn) bioaccumulation in the earthworm *Eisenia andrei* under different scenarios of climate change. Environ. Poll. 215, 178–186. http://dx.doi.org/10.1016/].envpol.2016.05.012.

González-Alcaraz, N.M., van Gestel, C.A.M., 2016b. Toxicity of a metal(|oid)-polluted agricultural soil to *Enchytraeus crypticus* changes under a global warming perspective: variations in air temperature and soil moisture content. Sci. Total Environ. 573, 203–211. http://dx.doi.org/10.1016/j.scitotenv.2016.08.061.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S—transferase: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Imfeld, G., Vuilleumier, S., 2012. Measuring the effects of pesticides on bacterial communities in soil: a critical review. Eur. J. Soil Biol. 49, 22–30. http://dx.doi.org/10.1016/j.ejsobi.2011.11.010.

IPCC, 2007. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, Climate Change 2007: Synthesis Report. IPCC, Geneva, Switzerland.

Jia, M., Cao, G., Li, Y., Tu, X., Wang, G., Nong, X., Whitman, D.W., Zhang, Z., 2016. Biochemical basis of synergism between pathogenic fungus *Metarhizium anisopliae* and insecticide chlorantraniliprole in *Locusta migratoria* (Meyen). Sci. Rep. 6, 28424. http://dx.doi.org/10.1038/srep28424.

Jiang, W., Duysen, E.G., Lockridge, O., 2012. Induction of plasma acetylcholinesterase activity and apoptosis in mice treated with the organophosphorus toxicant, tri-o-cresyl phosphate. Toxicol. Res. 1, 55–61. http://dx.doi.org/10.1039/C2TX20009K.

Kalam, A., Mukherjee Asok, K., 2002. Effects of hexaconazole, propiconazole, profenofos and pretilachlor on soil phosphatase and dehydrogenase activities. Pestic. Res. J. 14, 337–342.

Kattwinkel, M., Kiihne, I.-V., Foit, K., Liess, M., 2011. Climate change, agricultural insecticide exposure, and risk for freshwater communities. Ecol. Appl. 21 (6), 2068–2081.

Khan, M.A.Q., Ahmed, S.A., Salazar, A., Gurumendi, J., Khan, A., Vargas, M., Von Catalin, B., 2007. Effect of temperature on heavy metal toxicity to earthworm *Lumbricus terrestris* (Annelida: oligochaeta). Environ. Toxicol. 22, 487–494.

Khessiba, A., Roméo, M., Aïssa, P., 2005. Effects of some environmental parameters on catalase activity measured in the mussel (*Mytilus galloprovincialis*) exposed to lindane. Environ. Poll. 133, 275–281.

Koleva, N.G., Schneider, U.A., 2009. The impact of climate change on the external cost of pesticide applications in US agriculture. Int. J. Agric. Sustain. 7, 203–216.

Konwick, B.J., Garrison, A.W., Avants, J.K., Fisk, A.T., 2006. Bioaccumulation and biotransformation of chiral triazole fungicides in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 80, 372–381.

Kookana, R., Holz, G., Barnes, C., Bubb, K., Fremlin, R.R., Boardman, B., 2010. Impact of climatic and soil conditions on environmental fate of atrazine used under plantation forestry in Australia. J. Environ. Manag. 91, 2649–2656.

Larson, J.L., Redmond, C.T., Potter, D.A., 2012. Comparative impact of an anthranilic diamide and other insecticidal chemistries on beneficial invertebrates and ecosystem services in turfgrass. Pest Manag. Sci. 68, 740–748.

Laskowski, R., Bednarska, A.J., Kramarz, P.E., Loureiro, S., Scheil, V., Kudłek, J., Holmstrup, M., 2010. Interactions between toxic chemicals and natural environmental factors e a meta-analysis and case studies. Sci. Total Environ. 408, 3763–3774.

Lavtižar, V., Berggren, K., Trebše, P., Kraak, M.H., Verweij, R.A., van Gestel, C.A., 2016. Comparative ecotoxicity of chlorantraniliprole to non-target soil invertebrates. Chemosphere 159, 473–479. http://dx.doi.org/10.1016/j.chemosphere.2016.06.036.

Li, Y., Mao, M., Li, Y., Xiong, L., Li, Z., Xu, J., 2011. Modulations of high-voltage activated Ca^{2+} channels in the central neurones of *Spodoptera exigua* by chlorantraniliprole. Physiol. Entomol. 36, 230–234.

Li, Z.-H., Zlabek, V., Grabic, R., Li, P., Machova, J., Velisek, J., Randak, T., 2010a. Effects of exposure to sublethal propiconazole on the antioxidant defense system and Na^{+}-K^{+}-ATPase activity in brain of rainbow trout Oncorhynchus mykiss. Aquat. Toxicol. 98, 297–303.

Li, Z.-H., Zlabek, V., Grabic, R., Li, P., Randak, T., 2010b. Modulation of glutathione-related antioxidant defense system of fish chronically treated by the fungicide propiconazole. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152, 392–398.

Lima, M.P.R., Soares, A.M.V.M., Loureiro, S., 2011. Combined effects of soil moisture and carbaryl to earthworms and plants: simulation of flood and drought events. Environ. Pollut. 159, 1844–1851.

Lima, M.P.R., Cardoso, D.N., Soares, A.M.V.M., Loureiro, S., 2015. Carbaryl toxicity prediction to soil organisms under high and low temperature regimes. Ecotoxicl. Environ. Saf. 114, 263–272. http://dx.doi.org/10.1016/j.ecoenv.2014.04.004.

Liu, Y.-J., Zaprasis, A., Liu, S.-J., Drake, H.L., Horn, M.A., 2011. The earthworm *Aporrectodea caliginosa* stimulates abundance and activity of phenoxyalkanoic acid herbicide degraders. ISME J. 5, 473–485.

Long, S.M., Reichenberg, F., Lister, L.J., Hankard, P.K., Townsend, J., Mayer, P., Wright, J., Holmstrup, M., Svendsen, C., Spurgeon, D.J., 2009. Combined chemical (Fluoranthene) and drought effects on Lumbricus rubellus demonstrate the applicability of the independent action model for multiple stressor assessment. Environ. Toxicol. Chem. 28, 629–636. http://dx.doi.org/10.1897/08-187.1.

Lydy, M.J., Belden, J.B., Ternes, M.A., 1999. Effects of temperature on the toxicity of M-parathion, chlorpyrifos, and pentachlorobenzene to *Chironomus tentans*. Arch. Environ. Contam. Toxicol. 37 (4), 542–547.

Martikainen, E.A.T., Krogh, P.H., 1999. Effects of soil organic matter content and temperature on toxicity of dimethoate to *Folsomia fimetaria* (Collembola: isotomiidae). Environ. Toxicol. Chem. 18, 865–872.

Mrdaković, M., Ilijin, L., Vlahović, M., Matić, D., Gavrilović, A., Mrkonja, A., Perić-Mataruga, V., 2016. Acetylcholinesterase (AChE) and heat shock proteins (Hsp70) of gypsy moth (*Lymantria dispar* L.) larvae in response to long-term fluoranthene exposure. Chemosphere 159, 565–569. http://dx.doi.org/10.1016/j.chemosphere.2016.06.059.

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: environmental contaminants in a warming world. Environ. Int. 35, 971–986.

Nwani, C.D., Lakra, W.S., Nagpure, N.S., Kumar, R., Kushwaha, B., Srivastava, S.K., 2010. Toxicity of the herbicide atrazine: effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa punctatus* (Bloch). Int. J. Environ. Res. Public Health 7, 3298–3312.

O'Toole, G.A., 2011. Microtiter dish biofilm formation assay. J. Vis. Exp. http://dx.doi.org/10.3791/2437.

OECD, 1984. Guideline for Testing of Chemicals, No. 207, Earthworm Acute Toxicity Test. Organization for Economic Co—Operation and Development, Paris, France.

Petric, I., Karpouzas, D.G., Bru, D., Udikovic-Kolic, N., Kandeler, E., Djuric, S., Martin-Laurent, F., 2016. Nicosulfuron application in agricultural soils drives the selection towards NS-tolerant microorganisms harboring various levels of sensitivity to nicosulfuron. Environ. Sci. Pollut. Res. 23, 4320–4333. http://dx.doi.org/10.1007/s11356-015-5645-6.

Puurtinen, H.M., Martikainen, E.A.T., 1997. Effect of soil moisture on pesticide toxicity to an enchytraeid worm, *Enchytraeus* sp. Arch. Environ. Contam. Toxicol. 33, 34–41. http://dx.doi.org/10.1007/s002449900220.

Qi, S., Wang, C., Chen, X., Qin, Z., Li, X., Wang, C., 2013. Toxicity assessments with Daphnia magna of Guadipyr, a new neonicotinoid insecticide and studies of its effect on acetylcholinesterase (AChE), glutathione S-transferase (GST), catalase (CAT) and chitobiase activities. Ecotoxicol. Environ. Saf. 98, 339–344. http://dx.doi.org/10.1016/j.ecoenv.2013.09.013.

R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. ⟨https://www.R-project.org/⟩.

Ramudu, A.C., Mohiddin, G.J., Srinivasulu, M., Madakka, M., Rangaswamy, V., 2011. Impact of Fungicides Chlorothalonil and Propiconazole on Microbial Activities in Groundnut (*Arachis hypogaea* L.) Soils. ISRN Microbiol. 2011, 1–7. http://dx.doi.org/10.5402/2011/623404.

Ratnakar Reddy, K., Poornachandra, Y., Jitender Dev, G., Mallareddy, G., Nanubolu, J.B., Kumar, C.G., Narsaiah, B., 2015. Synthesis of novel amide functionalized 2H-chromene derivatives by Ritter amidation of primary alcohol using HBF4·OEt2 as a mild and versatile reagent and evaluation of their antimicrobial and anti-biofilm activities. Bioorg. Med. Chem. Lett. 25, 2943–2947. http://dx.doi.org/10.1016/j.bmcl.2015.05.041.

Ricart, M., Barceló, D., Geiszinger, A., Guasch, H., Alda, M.L., de, Romaní, A.M., Vidal, G., Villagrasa, M., Sabater, S., 2009. Effects of low concentrations of the phenylurea herbicide diuron on biofilm algae and bacteria. Chemosphere 76, 1392–1401. http://dx.doi.org/10.1016/j.chemosphere.2009.06.017.

Ricart, M., Guasch, H., Alberch, M., Barceló, D., Bonnineau, C., Geiszinger, A., Farré, M., Ferrer, J., Ricciardi, F., Romaní, A.M., Morin, S., Proia, L., Sala, L., Sureda, D., Sabater, S., 2010. Triclosan persistence through wastewater treatment plants and its potential toxic effects on river biofilms. Aquat. Toxicol. 100, 346–353. http://dx.doi.org/10.1016/j.aquatox.2010.08.010.

Rodrigues, A.C., Gravato, C., Quintaneiro, C., Golovko, O., Žlábek, V., Barata, C., Soares, A.M., Pestana, J.L., 2015. Life history and biochemical effects of chlorantraniliprole on *Chironomus riparius*. Sci. Total Environ. 508, 506–513. http://dx.doi.org/10.1016/j.scitotenv.2014.12.021.

Römbke, J., Garcia, M.V., Scheffczyk, A., 2007. Effects of the fungicide benomyl on earthworms in laboratory tests under tropical and temperate conditions. Arch. Environ. Contam. Toxicol. 53, 590–598.

Roy, C., Gaillardon, P., Montfort, F., 2000. The effect of soil moisture content on the sorption of five sterol biosynthesis inhibiting fungicides as a function of their physicochemical properties. Pest Manag. Sci. 56, 795–803.

RStudio Team, 2016. RStudio: Integrated Development for R. RStudio, Inc, Boston, MA. 〈http://www.rstudio.com/〉.

Sanchez-Hernandez, J.C., Martínez Morcillo, S., Notario del Pino, J., Ruiz, P., 2014. Earthworm activity increases pesticide-sensitive esterases in soil. Soil Biol. Biochem. 75, 186–196. http://dx.doi.org/10.1016/j.soilbio.2014.04.015.

Shaikh, H.M., More, B.C., Patel, N.G., 2016. Effect of sub lethal doses of Coragen and 2,4-D ethyl ester on digestive enzymes of earthworm species *Eudrilus eugeniae*, Kienberg 1867. Int. J. Innov. Res. Sci. Eng. Technol. 5 (12), 21174–21177. http://dx.doi.org/10.15680/IJIRSET.2016.0512046.

Stepić, S., Hackenberger, B.K., Velki, M., Lončarić, Ž., Hackenberger, D.K., 2013. Effects of individual and binary-combined commercial insecticides endosulfan, temephos, malathion and pirimiphos-methyl on biomarker responses in earthworm *Eisenia andrei*. Environ. Toxicol. Pharmacol. 36, 715–723. http://dx.doi.org/10.1016/j.etap.2013.06.011.

Tabassum, H., Khan, J., Salman, M., Raisuddin, S., Parvez, S., 2016. Propiconazole induced toxicological alterations in brain of freshwater fish *Channa punctata* Bloch. Ecol. Indic. 62, 242–248.

Tarradellas, J., Bitton, G., Rossel, D. (Eds.), 1996. Soil Ecotoxicology. CRC Press, New York.

Tu, H.T., Silvestre, F., De Meulder, B., Thome, J.-P., Phuong, N.T., Kestemont, P., 2012. Combined effects of deltamethrin, tempearture and salinity on oxidative stress biomarkers and acetylcholinesterase activity in the blck tiger shrimp (Penaeus monodon). Chemosphere 86, 83–91. http://dx.doi.org/10.1016/j.chemosphere.2011.09.022.

Tubiello, F.N., Rosenzweig, C., Goldberg, R.A., Jagtap, S., Jones, J.W., 2002. Effects of climate change on US crop production: simulation results using two different GCM scenarios. Part I: wheat, potato, maize, and citrus. Clim. Res. 20 (3), 259–270.

Uvarov, A.V., Tiunov, A.V., Scheu, S., 2011. Effects of seasonal and diurnal temperature fluctuations on population dynamics of two epigeic earthworm species in forest soil. Soil Biol. Biochem. 43, 559–570. http://dx.doi.org/10.1016/j.soilbio.2010.11.023.

Van den Berg, F., Kubiak, R., Benjey, W.G., Majewski, M.S., Yates, S., Reeves, G.L., 1999. Emission of pesticides into the air. Water Air Soil Pollut. 115, 195–218.

Wang, J., Wang, J., Xu, C., Liu, R., Chen, Y., 2016. Molecular mechanism of catalase activity change under sodium dodecyl sulfate-induced oxidative stress in the mouse primary hepatocytes. J. Hazard Mater. 307, 173–183. http://dx.doi.org/10.1016/j.jhazmat.2015.11.060.

Wang, Z.H., Yang, T., Qin, D.M., Yong, G., Ying, J., 2008. Determination and dynamics of difenoconazole residues in Chinese cabbage and soil. Chin. Chem. Lett. 19 (8), 969–972.

Wolińska, A., Stępniewska, Z., 2012. Dehydrogenase activity in the soil environment. In: Canuto, R.A. (Ed.), Dehydrogenases. InTech, pp. 183–210. http://dx.doi.org/10.5772/48294. https://www.intechopen.com/books/dehydrogenases/dehydrogenase-activity-in-the-soil-environment.

Yen, J.-H., Chang, J.-S., Huang, P.-J., Wang, Y.-S., 2009. Effects of fungicides triadimefon and propiconazole on soil bacterial communities. J. Environ. Sci. Health Part B 44, 681–689. http://dx.doi.org/10.1080/03601230903163715.

Zhang, X., Yang, F., Zhang, X., Xu, Y., Liao, T., Song, S., 2008. Induction of hepatic enzymes and oxidative stress in Chinese rare minnow (*Gobiocypris rarus*) exposed to waterborne hexabromocyclododecane (HBCDD). Aquat. Toxicol. 86, 4–11.

Zhang, X.J., Yang, L., Zhao, Q., Caen, J.P., He, H.Y., Jin, Q.H., Guo, L.H., Alemany, M., Zhang, L.Y., Shi, Y.F., 2002. Induction of acetylcholinesterase expression during apoptosis in various cell types. Cell Death Differ. 9, 790–800. http://dx.doi.org/10.1038/sj.cdd.4401034.